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STEREOSPECIFICITY OF THE CATALYSIS OF GF BY THE ORGANOPHOSPHORUS ACID ANHYDROLASE AND PHOSPHOTRIESTERASE ENZYMES

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13. ABSTRACT *(Maximum 200 words)*
The stereospecificity of the catalysis of GF hydrolysis by several enzymes was investigated. Stereospecificity was not evident at higher (3 mM) GF concentrations but was clearly apparent at 0.5 mM GF. The differential effect was due to fluoride-catalyzed racemization of the substrate. The enzymes JD6.5 organophosphorus acid anhydrolase (OPAA), *Alteromonas haloplanktis* OPAA, and the wild-type phosphotriesterase (PTE) enzymes were all found to preferentially catalyze the hydrolysis of the (+)GF isomer, as determined by GC analysis of the remaining isomer. Acetylcholinesterase inhibition experiments showed the purified (-)GF isomer to be approximately twice as toxic as the racemic compound. One PTE mutant, H254G/H259W/L303T, was found to preferentially catalyze the hydrolysis of the (-)GF isomer, as shown by its complementation of JD6.5 OPAA and subsequent GC analysis of the remaining (+)GF isomer. Thus, either GF isomer could be individually prepared enzymatically and analyzed isothermally on a GC.

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PREFACE

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STEREOSPECIFICITY OF THE CATALYSIS OF GF BY THE ORGANOPHOSPHORUS ACID ANHYDROLASE AND PHOSPHOTRIESTERASE ENZYMES

1. INTRODUCTION

The enzyme organophoshorus acid anhydrolase (OPAA), from *Alteromonas sp.* JD6.5 has been shown to catalyze the hydrolysis of a number of toxic organophosphorus compounds including several G-type chemical nerve agents.[1,2] The enzyme has been cloned into *Escherichia coli* and can be produced at concentrations up to 300 mg per liter of culture, corresponding to approximately 50% of the total cellular protein.[3] A similar OPAA enzyme from *Alteromonas haloplanktis* has also been cloned and expressed in *E. coli.*[4]

The phosphotriesterase enzyme (PTE) has catalytic properties similar to OPAA in addition to some catalytic activity against VX. The PTE gene was found in nature on plasmids in both *Pseudomonas diminuta* MG and *Flavobacterium sp.* ATCC 27551 bacteria.[5,6,7] The gene has been cloned, sequenced[6-8] and over-expressed in several systems.[9-11] PTE catalyzes the hydrolysis of a broad spectrum of organophosphorus compounds including those with P-O, P-F, P-CN and P-S bonds to their leaving groups.[9,12-15] Recently, a number of site-directed mutants derived from PTE have also been characterized with respect to their activity against various organophosphate compounds.[16-18]

While considerable work has been published on the initial rate kinetics of these enzymes on various chemical agent substrates, relatively little attention has been paid to their stereospecificity, which is an important consideration for at least two reasons. First, it is possible that differential activity on stereoisomers could affect the overall enzymatic detoxification rate. For that reason, it needs to be determined whether all (or at least the most toxic) stereoisomers of a particular chemical agent are effective substrates for these enzymes. Second, it is possible that enzymatic stereospecificity could be exploited to produce higher value products through synthetic routes. Stereoselective enzymatic synthesis is a research area of considerable interest, for which John Cornforth was awarded a Nobel Prize in 1975.[19] Single enantiomer compounds play a role of great importance as biologically active compounds. The stereoselective and substrate-specific nature of enzymes makes them a good choice as catalysts in the synthesis of pharmaceuticals and other fine chemicals. Since the OPAA and PTE enzymes used in this study have been cloned, produced and purified in quantity, it was feasible to study their stereospecific interaction with various substrates.

Nerve agents and enzymes all exert their effects in a biological, chiral environment, so it should be expected that those effects would be stereoselective. This was first reported in 1955 by Michel,[20] who observed a biphasic inhibition of acetylcholinesterase (AChE) with GB. It is currently known that the chemical nerve

agents GD (*o*-pinacolyl methylphosphonofluoridate), GB (*o*-isopropyl methylphosphonofluoridate), GA (ethyl N,N-dimethylphosphoramidocyanidate) and VX (o-ethyl-S-[2-diisopropylaminoethyl] methylphosphonothiolate) all bind AChE stereospecifically resulting in a significant difference in toxicity between their respective enantiomers (Table).

Table. Toxicity of GB, GD, VX and GA isomers

Compound	**Isomer(s)**	**LD_{50} (mouse, μg/kg)**	**Reference**
GB	P(-)	41	Boter et al., *Biochem. Pharmacol.* 1969, *18*, 2403-2407
GB	Racemic	83	Van De Meent et al., TNO, unpublished results, 1987
VX	P(-)	12.6	(Hall et al., *J. Pharm. Pharmacol.* 1977, *29*, 574-576
VX	P(+)	165	Hall et al., *J. Pharm. Pharmacol.* 1977, *29*, 574-576
VX	Racemic	20.1	Van De Meent et al., TNO, unpublished results, 1987
GD	C(+)P(-)	99	Benschop et al., *Toxicol. Appl. Pharmacol.* 1984, *72*, 61-74
GD	C(-)P(-)	38	Benschop et al., *Toxicol. Appl. Pharmacol.* 1984, *72*, 61-74
GD	C(+)P(+)	>5000	Benschop et al., *Toxicol. Appl. Pharmacol.* 1984, *72*, 61-74
GD	C(-)P(+)	>2000	Benschop et al., *Toxicol. Appl. Pharmacol.* 1984, *72*, 61-74
GD	Racemic	156	Benschop et al., *Toxicol. Appl. Pharmacol.* 1984, *72*, 61-74
GA	P(-)	119	Degenhardt et al., *J. Am. Chem. Soc.* 1986, *108*, 8290-8291
GA	P(+)	837	Degenhardt et al., *J. Am. Chem. Soc.* 1986, *108*, 8290-8291
GA	Racemic	308	Degenhardt et al., *J. Am. Chem. Soc.* 1986, *108*, 8290-8291

GF is not listed in the table because the toxicity of individual GF isomers has not been reported in the literature. However, the material safety data sheet for GF (Edgewood Arsenal Special Report EO-SR-74002, December 1974, available from Defense Technical Information Center as AD C014 792) lists the intravenous toxicity of GF to rats at 53.0 μg/kg, to rabbits as 15.3 μg/kg and to goats as 9 μg/kg for the racemic material. Thus, the toxicity of racemic GF is comparable to that of GB and GD.

The known toxicities of G-agent and VX isomers can be summarized as follows:

- GD: The P(-) isomers account for essentially all of the compound's toxicity.

- GB: P(-) GB is approximately twice as toxic as racemic GB, indicating that essentially all the toxicity is derived from the P(-) isomer.

- VX: The (-) isomer is approximately 13 times more toxic than the (+) isomer.

- GA: The (-) isomer is approximately 7 times more toxic than the (+) isomer.

- GF: Toxicity of individual stereoisomers has not previously been reported.

2. MATERIALS AND METHODS

2.1 Enzyme Assays.

Enzyme assays were conducted with a fluoride electrode attached to a Fisher Accumet 925 meter. Reactions were conducted in a temperature-controlled vessel in a total volume of 2.5 mL. Buffering was provided by 50 mM bis-tris-propane at pH 7.2. $MnCl_2$ was added to the buffer to a final concentration of 1 mM for OPAA assays only (Mn^{2+} activates OPAA).

2.2 Gas Chromatography Method for Separation of GF Isomers.

A Hewlett-Packard model 6890 gas chromatograph equipped with a flame photometric detector in the phosphorus mode and a 25 m X 250 μm id X 0.12 μm Chirasil-Val--L column (Chrompack) was used to analyze the GF stereoisomers. The oven temperature was 90 °C (isothermal) and the inlet and detector temperatures both were 200 °C. The injection volume was 1.0 μL with a 100:1 split ratio. The carrier gas was helium with a 1 mL/min flow rate. Under these conditions the (-)GF and (+)GF peaks were separated by 0.15 min at a retention time of approximately 8 min.

2.3 Polarimetry.

Specific rotation of the single GF isomer was calculated based on measurements of the observed rotation made at 589 nm (sodium line) using a Perkin Elmer 141 Electronic Polarimeter and a sample cell with a path length of 10 cm.

2.4 AChE Inhibition Assays.

AChE enzyme inhibition assays were conducted essentially as described previously.[20] 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) was obtained from Aldrich Chemical Co. (Milwaukee, WI). DTNB (0.01 M) was dissolved in 100 mM potassium phosphate buffer pH 7.0. AChE was purchased from Sigma Chemical Co. (St. Louis, MO). Acetylthiocholine (a chromagenic AChE substrate) was purchased from Aldrich as an iodide salt and was 98% pure.

AChE solutions were made up to 10 ng/mL in water and split into two fractions. To one fraction was added 1 µL of a 10^{-9} dilution of GF (either racemic or the chromatographically-pure single isomer) in isopropanol. The other fraction (uninhibited) received no GF. Aliquots were removed in triplicate from both fractions and analyzed for their activity on acetylthiocholine by spectrophotometric measurements at 405 nm.

2.5 Construction of Mutant PTE Strain.

The site-directed mutant PTE strain was constructed using the method of overlap extension[22] and cassette insertion with a synthetic gene (Wohlschlegel, personal communication).

2.6 Enzyme Preparation.

The JD6.5 OPAA enzyme was prepared as described elsewhere.[23] Briefly, the *Escherichia coli* host cell containing the cloned OPAA gene was grown to late log phase in 1 L of LB broth in a bioreactor. Cells were harvested and the enzyme was purified by ammonium sulfate fractionation. The 40-65% ammonium sulfate pellet was redissolved, dialyzed and loaded onto a 10 mL Q Sepharose column. The enzyme was eluted from the column with a linear gradient of 0.2 to 0.6 M NaCl. Subsequent polyacrylamide gel electrophoresis of the pooled active protein peaks showed a single band.

Cells of *Escherichia coli* XL1 strain harboring pVSEOP7 (unpublished results) were used to purify the phosphotriesterase (PTE) enzyme. The pVSEOP7 contains a full-length opd gene cloned in the expression vector, pSE420 (Invitrogen Corp., CA, USA).

The cells were grown in Luria-Bertani broth in 6-L Erlenmeyer flasks containing 100 µg/mL ampicillin at 30 °C. The cells were grown to early log-phase (0.5 A_{600}), and induced with 0.6 mM IPTG. After induction, the cells were grown for an additional 14 hrs and 1 mM cobalt chloride was added. The cells were harvested four hrs after the addition of cobalt, by centrifugation and suspended in 10 mM bis-tris-propane, pH 7.8. Cells were frozen and stored at -40 °C before use. The cells were lysed by passing twice through a French press. The cell-free extract supernatant was collected following centrifugation at 15,000 rpm in a JA20 rotor for 45 min.

The native PTE enzyme was purified from the cell-free extract using a single strong cation exchange resin (unpublished protocol). The fractions containing the PTE activity were pooled, concentrated, and dialyzed against 10 mM BTP, pH 7.8 containing 50 µM cobalt chloride. The purified enzyme analyzed through native acrylamide gel electrophoresis (results not shown) was determined to be around 85-90% homogeneity. The protein content was determined using Coomassie Protein Assay Reagent (Pierce, Rockford, IL, USA) with BSA as a standard protein.

The *A. haloplanktis* OPAA enzyme was prepared from the American Type Culture Collection strain 23821.

3. RESULTS AND DISCUSSION

3.1 Assays - Theory.

Fluoride electrode assays offer a convenient means to determine whether there is differential activity on organophosphofluoridate stereoisomers. If defluorination activity is approximately similar on all stereoisomers, the plot of free (released) fluoride vs. time will approximate that obtained by base-mediated hydrolysis (equal activity on all isomers). If half the isomers are degraded significantly more rapidly than the others, there will be a midpoint deflection in the slope of the line (stereopreference). Based on these assumptions, approximate theoretical plots of released fluoride vs. time are illustrated in Figure 1.

3.2 JD6.5 OPAA Catalysis of Racemic GF: Differential Activity at 3 mM vs. 0.5 mM GF.

Figure 2 shows the results of JD6.5 OPAA catalysis of GF at a concentration of 3 mM. The monophasic curve is consistent with an enzyme that possesses similar activity on each of the two isomers.

Figure 3 shows the results of JD6.5 OPAA activity on GF at a concentration of 0.5 mM. In this instance, the curve exhibits a distinct midpoint deflection with the latter slope approaching that of the spontaneous rate. These results are consistent with substantially differential activity on the two stereoisomers and stand in marked contrast to the results observed with the higher (3 mM) GF concentration.

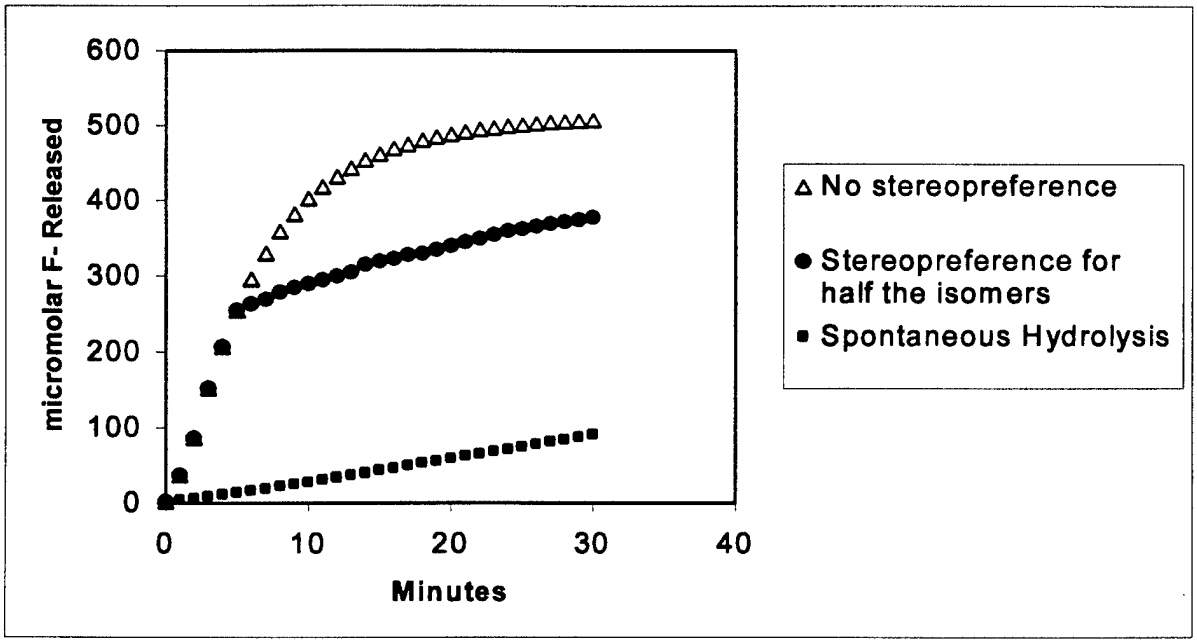


Figure 1. Theoretical curves illustrating the expected profiles of enzymes with similar activity on all isomers (no stereoselectivity), preferential activity on a single isomer (or half the isomers) and spontaneous hydrolysis. The curve without stereoselectivity shows no midpoint deflection whereas the stereoselectivity curve shows a midpoint deflection with the slope of the second half of the curve approaching that of the spontaneous hydrolysis rate.

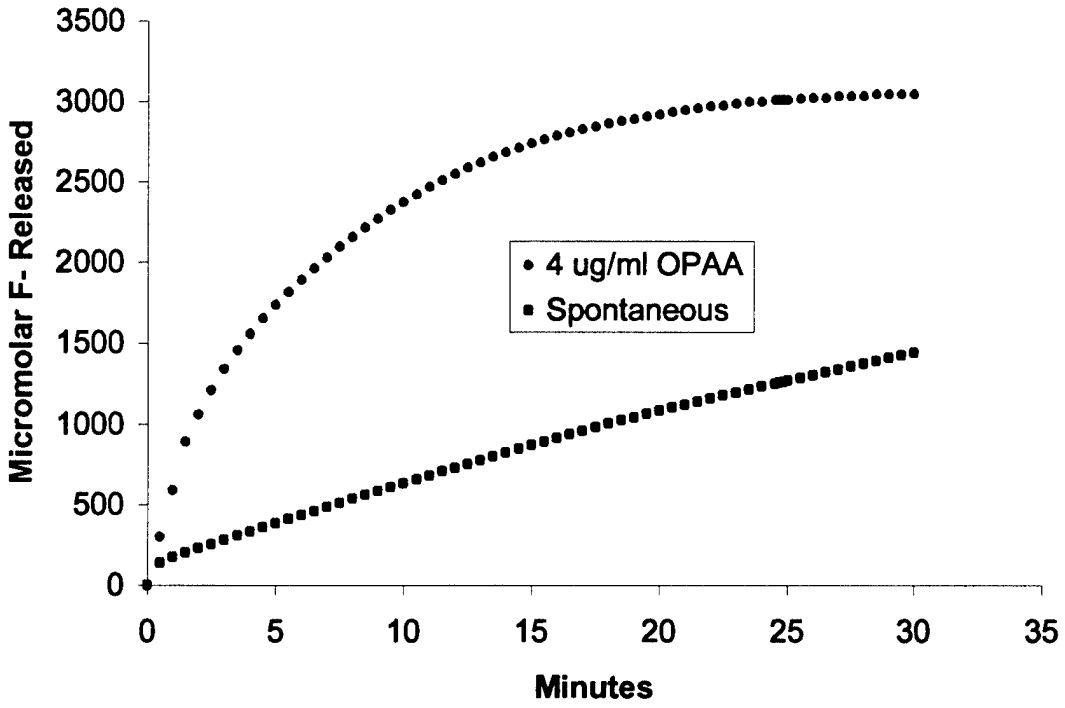


Figure 2. Activity profile of the JD6.5 OPAA enzyme with 3 mM GF. The curve is essentially monophasic, suggestive of similar activity on both GF isomers.

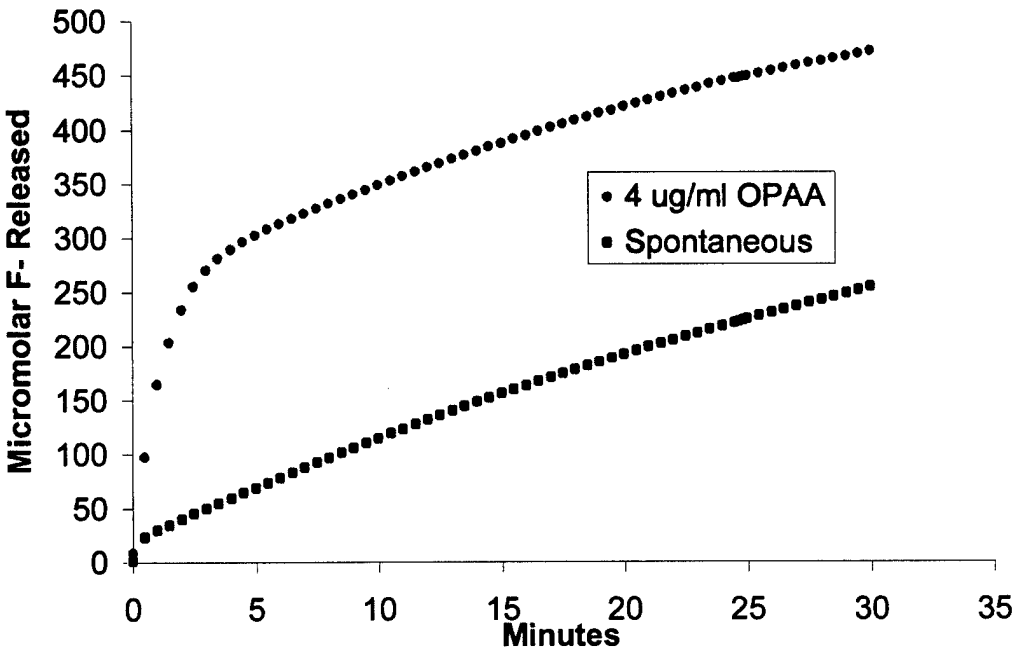


Figure 3. Activity profile of JD6.5 OPAA with 0.5 mM GF. The curve shows a distinct midpoint deflection, consistent with differential activity on the two isomers.

In 1965, Christen and Van den Muysenberg[24] observed a biphasic hydrolysis of low GB concentrations in rat plasma. When they did the same experiment with diisopropylfluorophosphate (a symmetrical molecule) the hydrolysis curve was monophasic. Also, when they did the experiment with higher GB concentrations the curve was similarly monophasic. Finally, when they added NaF to the plasma along with low concentrations of GB, the biphasic curve became monophasic. Their hypothesis was that fluoride had catalyzed the racemization of GB. As was expected, at the higher GB concentration, the fluoride released from the hydrolyzed GB was sufficient to cause racemization while at the lower GB concentration, racemization did not occur.

To determine if GF behaves similarly when catalyzed by JD6.5 OPAA, NaF was added to the enzymatic reaction containing 0.5 mM GF. Results (Figure 4) show an essentially monophasic curve, consistent with fluoride-catalyzed racemization of GF.

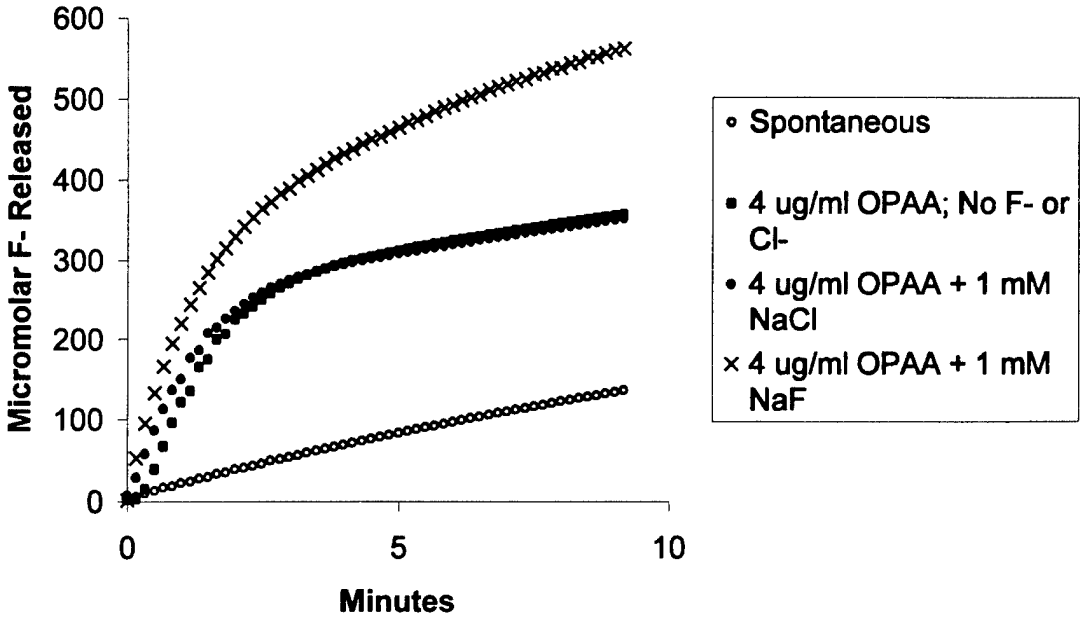


Figure 4. JD6.5 OPAA catalysis of 0.5 mM GF in the presence of 1.0 mM NaF or 1.0 mM NaCl. The profile of the reaction with NaCl is biphasic and almost identical to that with JD6.5 OPAA and GF alone, indicating that NaCl does not catalyze GF racemization. However, in the presence of 1 mM NaF, the reaction profile is almost monophasic, consistent with fluoride-catalyzed racemization of the GF substrate. The magnitude of the NaF trace is corrected for the exogenously added fluoride.

3.3 Gas Chromatographic Separation of GF Isomers.

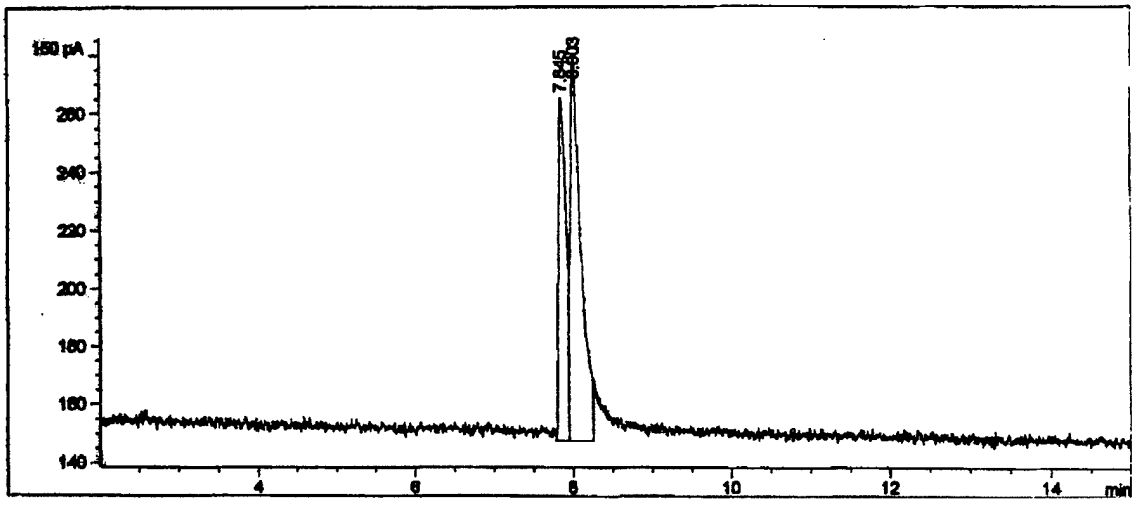
To distinguish the two GF stereoisomers, an isothermal GC method was developed (MATERIALS AND METHODS). The two GF stereoisomers were consistently separated by 0.15 to 0.2 min at retention times between eight and nine min, depending on the exact flow rate of the carrier gas. When racemic GF was injected, the area of the first peak was always slightly greater than half the area of the second peak (Figure 5.a).

3.4 Enzymatic Preparation and Polarimetry Analysis of a Single (-)GF Isomer.

A chromatographically pure (-)GF isomer was prepared by selectively degrading the isomer on which the enzyme had the greater activity. The JD6.5 OPAA enzyme reaction was run at 15 °C and pH 7.0 in order to minimize spontaneous hydrolysis. At approximately the midpoint deflection of the reaction profile, the solution was extracted with methylene chloride and the extract was analyzed by GC. A single GF isomer peak was observed with a retention time corresponding to the second GC peak (Figure 5.b).

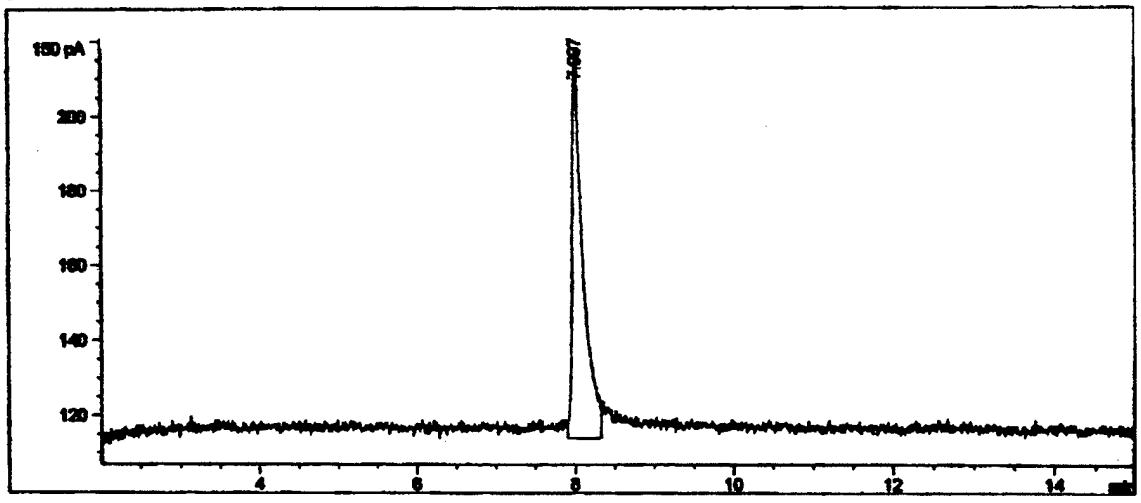
The single isomer preparation was concentrated approximately 10-fold by evaporation at room temperature and the optical rotation measurements were performed on a Perkin Elmer 141 electronic polarimeter (589 nm sodium line). The specific rotation was measured at –19.3°. Therefore, the enzymatic preparation is enriched for the (-)GF isomer, indicating the JD6.5 OPAA enzyme selectively degraded the (+)GF isomer.

a.



Peak #	Ret Time	Area %
1	7.845	37.40245
2	8.003	62.59755

b.

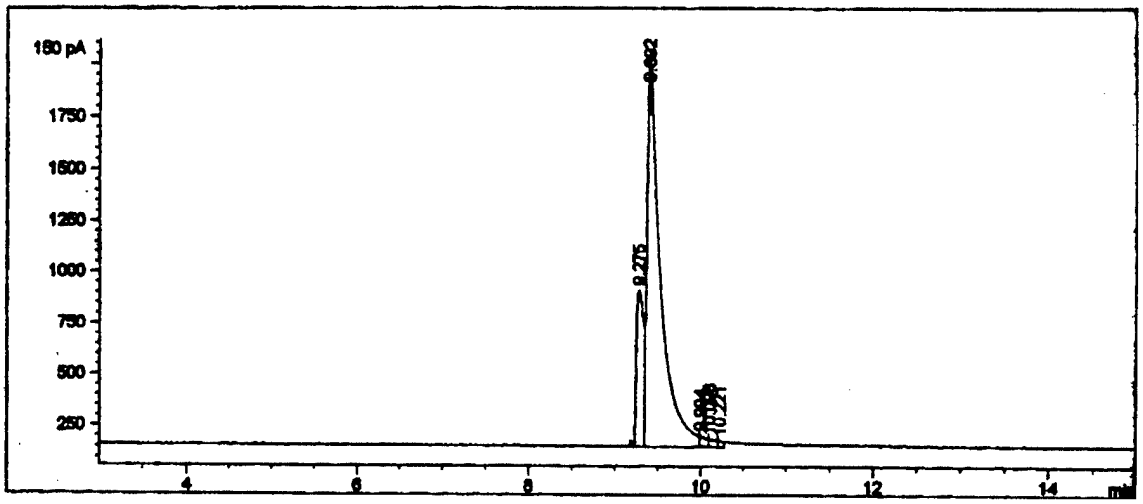


Peak #	Ret Time	Area %
1	7.997	1.000e2

Figure 5. a. Gas chromatogram of 90° isothermal separation of GF isomers.
b. GF following OPAA degradation of one isomer – only the second peak is still visible.

3.5 Fluoride-Catalyzed Racemization vs. Allosteric Alteration of the Enzyme.

Although the data in Figure 4 are consistent with fluoride-catalyzed racemization, an alternative explanation could be that fluoride was allosterically altering the enzyme and thereby changing its stereoselective properties. In this hypothetical case, the observed stereoselectivity would still occur only in the presence of sufficient concentrations of fluoride. In order to differentiate between these two possibilities, NaF was shaken with a solution of this single (-)GF isomer, with no enzyme present. Subsequent GC analysis showed two peaks, consistent with NaF catalyzed racemization of GF (Figure 6). Therefore, since the racemization occurs in the absence of enzyme, the direct effect of NaF on GF appears to be at least one means by which GF can be racemized.



Peak #	Ret Time	Area %
1	9.280	17.12859
2	9.398	81.69664
3	10.059	0.6050568
4	10.140	0.56909

Figure 6. Gas chromatogram of single (-)GF isomer to which NaF was added. The (+)GF peak has reappeared in the extract.

3.6 Complementation Test to Determine Stereospecificity of Three Enzymes.

Three different enzymes (JD6.5 OPAA, *A. haloplanktis* OPAA and wild-type PTE) were tested individually and together in order to compare their stereospecificity on 0.5 mM GF. As shown in Figure 7, the individual enzyme reactions all produce similarly biphasic profiles. A complementation test was used to determine if the enzymes were all acting on the same isomer or if one enzyme had activity on a different isomer than the other two. If two enzymes had significantly differential

stereospecificity then the shape of the curve should have tended towards monophasic. Experimentally, it was observed that the profile of the three enzyme reaction was essentially indistinguishable from the individual enzyme reaction profiles. Since little or no complementation was observed, it was evident that all three enzymes were primarily active on the same isomer. Since the polarimetry experiment established that the preference of the JD6.5 OPAA enzyme was for the (+)GF isomer, it can therefore be concluded that the PTE and *A. haloplanktis* OPAA enzymes also exhibit preferential activity on the (+) isomer.

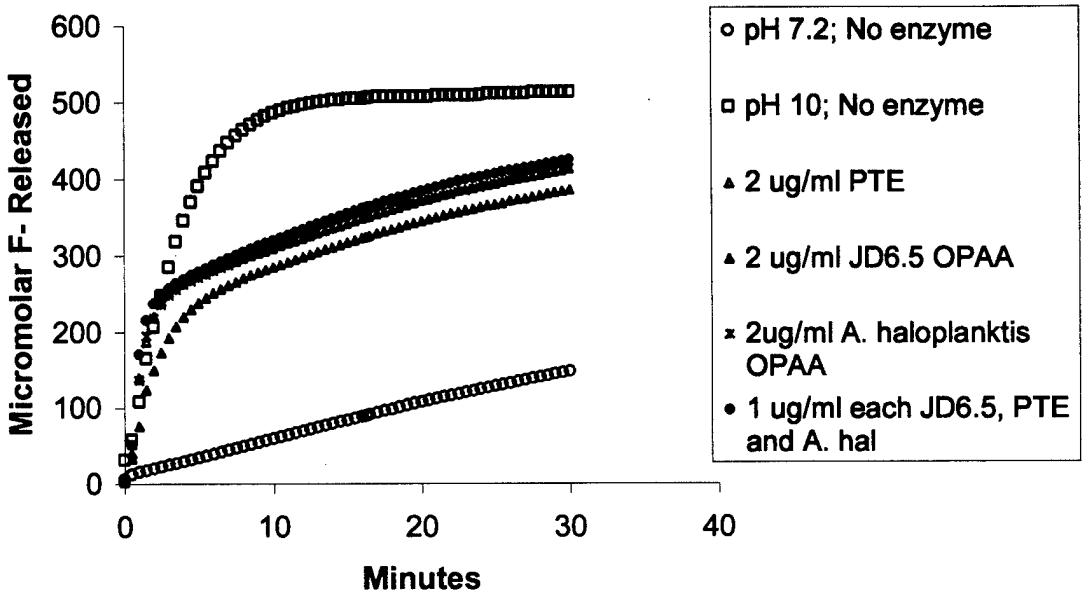


Figure 7. Biphasic fluoride release with PTE, JD6.5 OPAA and *A. haloplanktis* OPAA each alone and in combination. No complementation was evident, indicating that all three enzymes exhibited a marked preference for the same stereoisomer.

3.7 Stereospecific Reaction of GF in Whole Blood and Erythrocytes.

Whole human blood was drawn into tubes containing ethylenediaminetetraacetic acid (EDTA) to prevent clotting. To 5 mL of blood was added 1 mg of GF. Following a 15 min incubation at room temperature, the mixture was extracted with 1 mL of chloroform. The resulting extract was analyzed by GC and found to also be enriched for the (-)GF isomer. GF spiking and extraction of enriched human erythrocytes also yielded an enrichment of the (-)GF isomer. Although other explanations may be possible, the simplest explanation may be that the human paraoxonase enzyme in these samples exhibits the same stereoselectivity as the three bacterial enzymes examined earlier in this study.

3.8 AChE Inhibition of Respective GF Isomers.

The AChE inhibition was measured as a function of time in the presence of racemic GF, chromatographically-pure (-)GF, and without GF present (negative control). Since the (-) isomers of GA, GB, GD and VX are all much more toxic than the (+) isomers, the logical hypothesis was that the (-)GF isomer would inhibit AChE more strongly than racemic GF. If essentially all the toxicity is derived from a single isomer (as is the case with GB, for example), then the slope of the inhibition with (-)GF would be twice as steep as the inhibition with racemic GF. On the other hand, if essentially all the toxicity of GF is derived from the (+) isomer, the (-)GF inhibition slope would be half as steep as with racemic GF. If the two GF isomers are of similar toxicity, little or no change would be expected in the slope of the inhibition plot.

Figure 8 shows the results of the AChE inhibition assays that were performed in triplicate. Clearly, the (-)GF inhibits the AChE much more strongly than the racemic GF. Therefore, it is apparent that all three wild-type bacterial enzymes are preferentially degrading the less toxic isomer of GF.

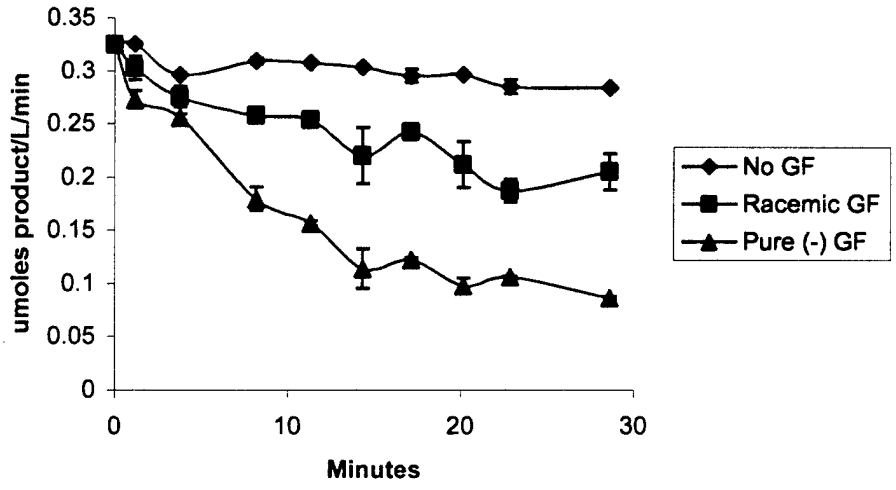


Figure 8. AChE inhibition by racemic and chromatographically pure (-)GF.

3.9 Stereospecificity of the PTE Mutant H254G/H259W/L303T (GWT).

In an attempt to identify an enzyme with preferential activity on the toxic GF isomer, a number of PTE mutants were screened. Most of these mutants showed either a marked preference for the non-toxic (+) isomer or had very low levels of activity overall. The PTE mutant H254G/H259W/L303T (GWT) was of particular interest since it was previously observed to have reversed stereospecificity on *p*-nitrophenyl

derivatives of G-type nerve agents (fluoride leaving group was substituted with a *p*-nitrophenyl group for colorimetric detection of the hydrolysis product). Figure 9 shows the hydrolysis profiles for the GWT mutant, JD6.5 OPAA and the two enzymes combined. Both enzymes alone exhibit a midpoint deflection and show complementation when combined. Therefore, their stereospecificity is opposite and the GWT mutant is preferentially catalyzing hydrolysis of the (-)GF isomer.

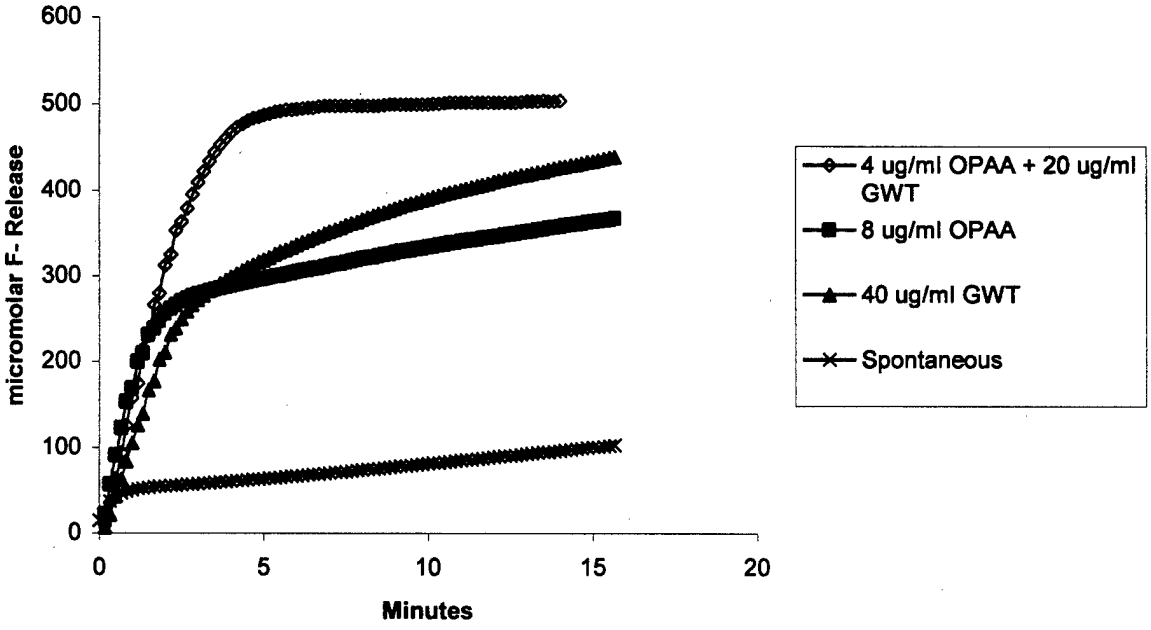


Figure 9. Complementation of GF stereochemistry: PTE mutant H254G/H259W/L303T (GWT) and JD6.5 OPAA.

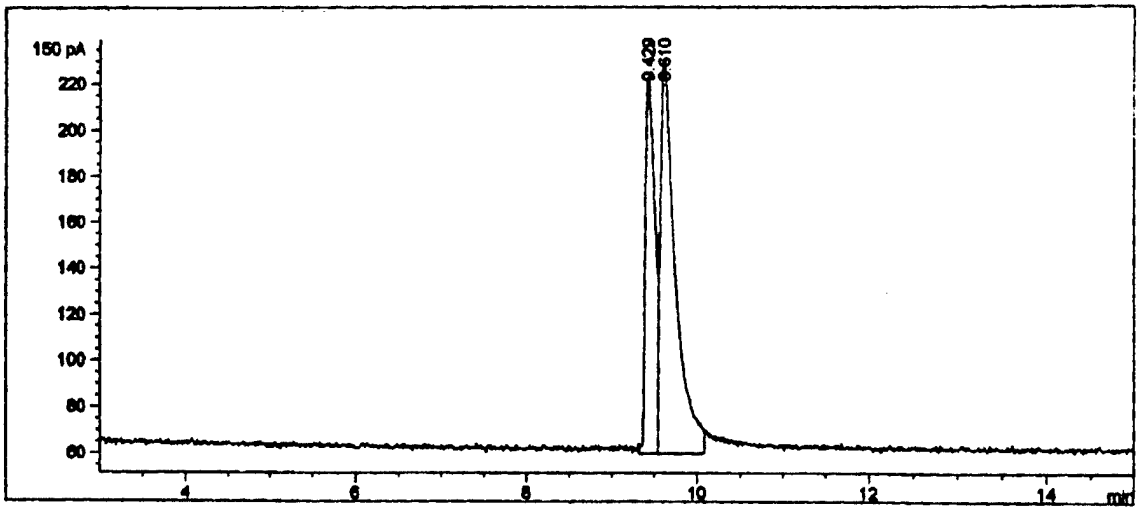
3.10 Enzymatic Preparation and Polarimetry Analysis of a Single (+)GF Isomer.

A chromatographically pure (+)GF isomer was prepared by selectively degrading the isomer on which the GWT enzyme had the greater activity (similar to the reaction conducted previously with the JD6.5 OPAA enzyme to produce the (-) isomer). At approximately the midpoint deflection of the reaction profile, the solution was extracted with methylene chloride and the extract was analyzed by GC. A single GF isomer peak was observed with a retention time corresponding to the first GC peak (Figure 10).

The single isomer preparation was concentrated approximately 10-fold by evaporation at room temperature and the optical rotation measurements were performed on a Perkin Elmer 141 electronic polarimeter (589 nm sodium line). The specific rotation was measured at +5°. Therefore, the enzymatic preparation is enriched

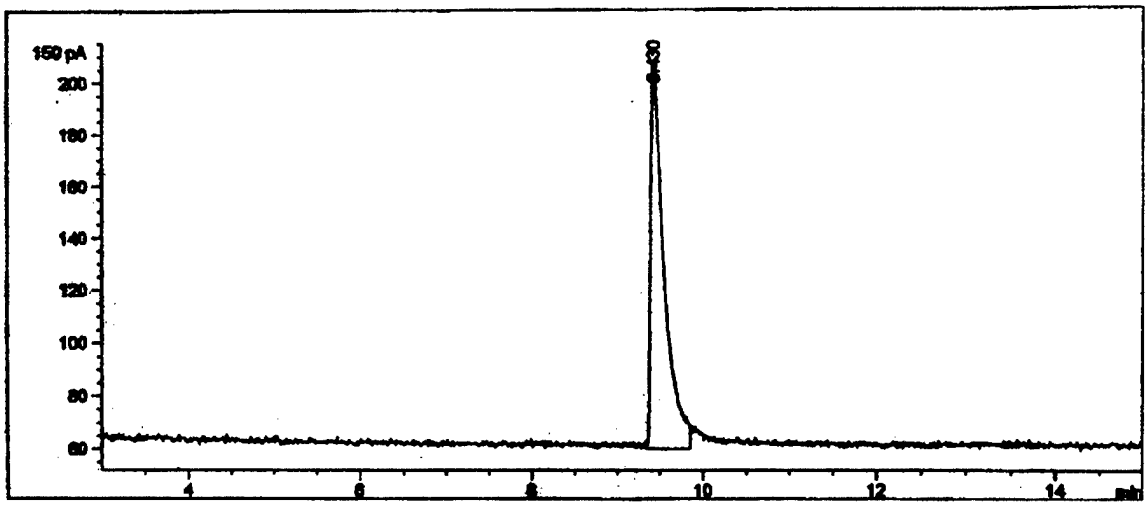
for the (+)GF isomer, indicating the GWT enzyme selectively degraded the (-)GF isomer.

a.



Peak #	Ret Time	Area %
1	9.429	37.34571
2	9.610	62.65429

b.



Peak #	Ret Time	Area %
1	9.430	1.000e2

Figure 10.a. Racemic GF prior to enzymatic degradation.
b. GF following enzymatic degradation of one isomer with the PTE mutant H254G/H259W/L303T. Only the first GC peak is still visible.

4. CONCLUSIONS

The JD6.5 OPAA enzyme was demonstrated to catalyze both GF stereoisomers at similar rates when the concentration of GF was 3 mM, whereas the same reaction with 0.5 mM GF showed marked stereospecificity, i.e., the plot of the release of free fluoride vs. time had a distinct midpoint deflection to a much lower slope.

When NaF is added to the enzymatic reaction, the 0.5 mM GF biphasic hydrolysis curve disappears. The same effect is not seen with the addition of NaCl to the enzymatic reaction. However, the addition of NaF to a chromatographically-pure single GF stereoisomer in the absence of enzyme causes racemization (the appearance of two peaks on the chromatogram). The results are similar to those observed previously with GB as the substrate, and are consistent with the fluoride-catalyzed racemization of GF at concentrations above 0.5 mM.

Both GF stereoisomers were resolved chromatographically, alone and together, using an isothermal GC method, described above.

A chromatographically-pure preparation of a single GF stereoisomer was made by extracting the OPAA catalysis reaction just past its midpoint deflection. Subsequent polarimetry analysis of the concentrated extract showed this stereoisomer to be (-)GF, with an optical rotation of -19.3°. This result indicates that the OPAA enzyme exhibits a preference for the (+)GF stereoisomer.

In addition to the JD6.5 OPAA enzyme, both the wild-type PTE and the *A. haloplanktis* OPAA were also shown to exhibit stereospecificity in their catalysis of 0.5 mM GF. Assays using a combination of the three enzymes together showed that neither of these latter two enzymes was able to complement the stereospecificity of JD6.5 OPAA. Thus, all three enzymes prefer the (+)GF stereoisomer.

Assays with whole human blood and with enriched erythrocytes also showed that the (+)GF stereoisomer was selectively degraded, since extracts showed an enrichment for (-)GF. The simplest explanation may be that human paraoxonase also exhibits a catalytic preference for the (+) isomer of GF.

The AChE inhibition of the purified (-)GF stereoisomer was approximately twice that of the racemic mixture, suggesting that the majority of the toxicity of GF is derived from the (-) stereoisomer. This is consistent with the other G and V-type nerve agents for which the (-) stereoisomer also represents most of the toxicity of the racemic compound.

A number of site-directed PTE mutants were screened for their activity against the (-)GF stereoisomer. Most mutants either had the same stereospecificity as the wild-type enzyme or had very little GF activity overall. One PTE mutant though (H254G/H259W/L303T), exhibited a marked catalytic preference for the (-)GF stereoisomer. This mutant enzyme was able to complement the activity of OPAA and

yielded chromatographically-pure (+)GF from an assay extracted just past the midpoint deflection. This mutant had previously been shown to have similar stereospecificity with the *p*-nitrophenyl derivative of GB (*p*-nitrophenyl isopropylmethylphosphonate).

Using chemical nerve agent degrading enzymes with varying stereospecificity, it was therefore possible to produce pure preparations of either GF stereoisomer by selectively degrading the other stereoisomer.

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